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Detection of *Babesia bigemina* in cattle of different genetic groups and in *Rhipicephalus* (*Boophilus*) *microplus* tick

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Abstract

Babesia bigemina infections were investigated in four genetic groups of beef cattle and in *Rhipicephalus* (*Boophilus*) *microplus* engorged female ticks. Blood samples and engorged female ticks were collected from 15 cows and 15 calves from each of the following genetic groups: Nelore, Angus × Nelore, Canchim × Nelore, and Simmental × Nelore. Microscopic examination of blood smears and tick hemolymph revealed that merozoites of *B. bigemina* (6/60) as well as kinetes of *Babesia* spp. (9/549) were only detected in samples (blood and ticks, respectively) originated from calves. PCR-based methods using primers for specific detection of *B. bigemina* revealed 100% infection in both calves and cows, regardless the genetic group. Tick infection was detected by nested-PCR amplifications showing that the frequency of *B. bigemina* was higher ($P < 0.01$) in female ticks collected from calves (134/549) than in those collected from cows (52/553). The frequency of *B. bigemina* was similar in ticks collected from animals, either cows or calves, of the four genetic groups ($P > 0.05$).

Keywords: Beef cattle; Tick; Babesiosis; PCR

1. Introduction

The *Rhipicephalus* (*Boophilus*) *microplus* and the tick-borne hemoparasites are one of the main economic constrains in Latin America livestock (Nari, 1995). The losses caused by ticks and the increasing spread of tick acaricide resistance have motivated research on alternative methods for controlling these arthropods. Among these methods, the utilization of resistant breeds is recognized as an economically feasible alternative to acaricides and other chemotherapeutics in tick and tick-borne pathogen controlling programs (Mattioli and Dempfle, 1995). The great resistance of zebu cattle to ticks and to babesiosis is a widely known phenomenon, which motivated replacement of pure *Bos taurus* by *Bos indicus* cattle and their crosses in areas of high tick infestation in Australia (Jonsson, 2006). According to this author, purebred *B. indicus* cattle will carry 10–20% as many ticks as pure *B. taurus* cattle, and crossbred cattle will carry somewhere in between, more or less proportional to their *B. indicus* content, when exposed to the same environmental conditions of infestation. Although the genes involved in this expression have not

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been identified, it is widely known that these phenotypic characteristics are hereditary.

In relation to tick-transmitted diseases, it is also accepted that pure *B. indicus* cattle present high levels of innate resistance to *Babesia bovis* and *Babesia bigemina* infections when compared to *B. taurus* and their crosses (Bock et al., 1997, 1999). This great resistance is manifested, for example, by lower parasitemia in *B. indicus* compared to *B. taurus* kept under identical pasture conditions (Aguirre et al., 1990). Besides that, percentage of *R. microplus* females infected when feeding on *B. indicus* cattle is significantly lower than that of parasites feeding on *B. taurus* (Guglielmone et al., 1989). Therefore, it was postulated that, in some situations, introduction of *B. indicus* blood would reduce the level of inoculation of *Babesia* to the point of converting an enzootic stability situation into instability (Guglielmone, 1995; Uilenberg, 1995), increasing the risk of babesiosis outbreak.

R. microplus occurs in all 26 Brazilian states (Lima et al., 2000) and its eradication is not feasible. In such situation, successful management of bovine babesiosis will be based on the knowledge of the interactions between *Babesia* parasites, their vector, and cattle host. In the present study, polymerase chain reaction (PCR)-based methods were used to assess the prevalence of *B. bigemina* in pure *B. indicus* and in three of *B. indicus* × *B. taurus* crosses (50% F1 generation) and in *R. microplus* female ticks that engorged in these animals, in order to verify if *B. indicus* genetics interferes with the transmissibility of *B. bigemina.*

2. Materials and methods

2.1. Animals

Animals used in this experiment belonged to a herd reared at Southeast Embrapa, located in São Carlos, SP, Brazil (latitude 22°01′ South, longitude 47°53′ West), with a subtropical climate. At this research center, there are 2800 bovines of the following breeds: Canchim, Nelore, and their crosses with European breeds. These animals are used in experiments destined to generate, adapt, and transfer applicable technologies to the Brazilian cattle ranching. Previous studies allowed the classification of this region as an endemic stable area for babesiosis (Oliveira et al., 2005; Oliveira-Sequeira et al., 2005).

Blood samples and *R. microplus* engorged female ticks were collected from 120 animals of four same-frame-sized genetic groups of beef cattle composed of 15 cows aged more than 3 years old, and 15 calves of 8–12 months of age. One group (N) was of pure breed *B. indicus* (Nelore) and the other three were of *B. indicus* × *B. taurus* (50% F1 generation) as follows: Nelore × Angus (A/N), Canchim × Nelore (C/N), and Simmental × Nelore (S/N). The animals were kept in paddocks naturally infested by *R. microplus* larvae and were left without tick chemical control for at least 40 days before the onset of the experimental observations and during all experimental period (February–March 2004), in order to avoid possible interference with the viability of ticks and of their offspring.

2.2. Samples collection and processing

Blood and tick samples were collected from February to March 2004. From each animal, a blood sample from the jugular vein and one from auricular vessels were taken for DNA extraction and blood smears, respectively. At the same time, a maximum of 10 standard-sized female ticks (≥4.5 mm) (Wharton and Utech, 1970) were collected from each animal.

Thin blood smears were stained with Giemsa for microscopic detection of *Babesia* and the assessment of parasitemia. Blood DNA was extracted from 300 μL of each sample using GFX™ kit (Genomic Blood DNA Purification kit, GE Health Care), as recommended by the manufacturer.

R. microplus females were individually incubated in BOD chambers at 27 ± 1 °C and 85–86% relative humidity for oviposition. Hemolymph from each individual female was sampled at the 18th day of incubation by removing a distal leg segment and blotting the exuding droplet of hemolymph on a slide (Burgdorfer, 1970). The slides were stained by Giemsa and examined under light microscope with a 100× objective. Kinetes were counted on 15 microscopic fields and results are reported in terms of mean kinetes/field. Next, each female tick was transferred to a labeled microtube and stored in a freezer at −80 °C, for later DNA extraction.

DNA extraction from *B. microplus* females was performed according to procedures described by Oliveira-Sequeira et al. (2005). Briefly, each frozen specimen was individually macerated in a microtube, 10 mL of buffer (10 mM Tris–HCl; 1 mM EDTA and 5% Triton X-100, pH 8.5) were added and the mixture was incubated for 15 min at room temperature. In sequence, 20 μL of proteinase K solution was added, and incubated in a water bath for 4 h at 56 °C, and for 10 min at 70 °C. After incubation, DNA extraction was performed using GFX™ kit protocol.

2.3. PCR and nested-PCR (nPCR)

B. bigemina DNA was amplified by PCR and/or nPCR, employing internal and external primers designed by Figueroa et al. (1993). All DNA samples extracted from ticks were submitted to amplification by nPCR whereas only negative PCR blood samples were submitted to this second amplification.

PCR was performed in a 25-μL solution containing 10 mM Tris–HCl; 50 mM KCl; 1.5 mM $MgCl_2$; 1.5 U Taq-DNA-polymerase (GE Health Care); 0.2 μM of each nucleotide (Amersham Bioscience); 10 pm of each primer and 5 mL of DNA samples. The same buffer at the same concentrations and 2 μL of the previously PCR amplified products were used for nPCR. Sensibility and specificity of primers, as well as conditions for reactions were determined previously (Oliveira et al., 2005; Oliveira-Sequeira et al., 2005).

Purified samples of *B. bigemina* (provided by Drs. Raul H. Kessler and Cláudio Madruga-Embrapa Beef Cattle, Brazil) were used as positive controls of PCR and nPCR reactions. A tube containing no DNA sample was included in each reaction batch for negative control.

PCR and nPCR amplified products were submitted to electrophoresis on 1.5% agarose gel. Samples presenting visible bands of 278 base pairs (PCR) or 170 base pairs (nPCR) were considered positive for *B. bigemina*. Amplicons sequence revealed correspondence to the sequences deposited in the GenBank under number S45366 (Spel–Aval restriction fragment of *B. bigemina*).

2.4. Statistical analysis

Chi-square and exact Fisher tests were used to analyze the frequency of *B. bigemina* infection in both cattle and ticks, in function of age and genetic group of bovines. All analyses were made utilizing FREQ protocol of SAS system of statistical analysis (SAS, 1996).

3. Results

3.1. Blood samples

Merozoites of *B. bigemina* (less than 0.1% of parasitized erythrocytes) were found exclusively in blood smears of six calves. Of the animals carrying merozoites, four were pure Nelore and two were Angus × Nelore, showing no association between presence of *B. bigemina* merozoites and the genetic constitution of the animals.

Data concerning to the PCR-based detection (PCR/nPCR) of *B. bigemina* in blood samples of calves and cows according to genetic group are presented in Table 1. There was no association between frequency of *B. bigemina* infection and genetic constitution or age of the animals ($P > 0.05$). DNA of *B. bigemina* was detected in all animals, but the number of PCR-positive calves (51/60) was significantly higher ($P < 0.01$) than the number of PCR-positive cows (9/60). However, when using the more sensitive nPCR test the blood samples of all remaining cattle and calves were found to be positive for *B. bigemina* DNA.

3.2. R. microplus samples

Kinetes of *Babesia* spp. were found exclusively in the hemolymph of female ticks engorged on calves (9/549). Average number of kinetes per microscopic field varied from 0.13 to 3.3. Of the nine females carrying kinetes, four had engorged on pure Nelore calves, one on Canchim × Nelore, one on Angus × Nelore and three on Simmental × Nelore. Frequency of kinetes in tick hemolymph by direct exam is too difficult to analyze, because this test is not sensitive and specific enough for utilization when concluding about B. *bigemina* vector infection rate. Natural infections of *R. microplus* with some species of *Babesia* were reported: *B. equi* and *B. caballi* (Battsetseg et al., 2002) and *B. bigemina* and *B. bovis* (Oliveira et al., 2005; Oliveira-Sequeira et al., 2005). Since determination of

Table 1
PCR and nPCR detection of *B. bigemina* in blood samples of calves and cows, according to their genetic group

	B. bigemina DNA							
	N		A/N		C/N		S/N	
	PCR	nPCR	PCR	nPCR	PCR	nPCR	PCR	nPCR
Calves	12	3	11	4	14	1	14	1
Cows	3	12	1	14	3	12	2	13
Total	15	15	12	18	17	13	16	14

Table 2
Frequency of *B. bigemina* infection in *R. microplus* female ticks engorged on calves ($n = 549$) and cows ($n = 553$) of different genetic groups*

	Infected female tick				
	N	A/N	C/N	S/N	Total
Calves	36/134 a	33/125 a	33/140 a	32/150 a	134/549 a
Cows	13/120 b	13/133 b	14/150 b	12/150 b	52/553 b
Total	49/254	46/258	47/290	44/300	186/1102

* Different letters in columns indicate significant difference.

this species cannot be done by kinetes morphologic analysis (Guglielmone, 1996), these data were not used.

Results of the nPCR for the detection of *B. bigemina* DNA in *R. microplus* females are presented in Table 2. Frequency of *B. bigemina* infection was significantly higher ($P < 0.01$) in ticks which fed on calves (24.4%) than in that of those which fed on cows (9.40%). There was no difference in frequency of *B. bigemina* infection in ticks related to the genetic groups ($P > 0.05$) of cattle.

4. Discussion

The region where the experiment was carried out is considered an area of enzootic stability, in which we previously demonstrated that crossbred calves of susceptible dairy herd become infected during their 1st week of life (Oliveira et al., 2005; Oliveira-Sequeira et al., 2005). Continuing those searches, one of the goals of this study was to investigate implications of different bovine genotypes on epidemiology of *B. bigemina*, especially concerning to resistant genotypes.

Enzootic stability is an epidemiological concept accepted for decades whereby the rate of transmission of *Babesia* is sufficient to infect the majority of calves before calfhood resistance is lost, between 6 and 9 months of age (Mahoney and Ross, 1972). Central to the theory and practice of endemic stability is that the disruption of this complex relationship might result in an outbreak of clinical babesiosis. Some authors considered that incorporating *B. indicus* genetics depresses the inoculation rate of *Babesia* and so areas with stability to babesiosis could be transformed into areas with instability (Mahoney et al., 1981; Guglielmone, 1995; Uilenberg, 1995). At least in respect to *B. bigemina*, data obtained in the present study do not support these assumptions, since infection was detected in all animals, regardless their age and genetic constitution. Besides the fact that frequencies of *B. bigemina* infections of pure *B. indicus* bovines and in *B. indicus* × *B. taurus* crosses did not differ, they were similar to the frequency previously detected in animals with predominantly *B. taurus* genetics (Oliveira et al., 2005; Oliveira-Sequeira et al., 2005), considered less resistant to ticks and babesiosis.

It is important to point out that the parameter most frequently used to classify zones of occurrence of *Babesia* has been the prevalence of specific antibodies (Araújo et al., 1997; Souza et al., 2000; Madruga et al., 2001; Barros et al., 2005). So, the low prevalence of specific antibodies against *B. bigemina,* previously detected in Nelore cattle in São Paulo state, Brazil (D'Andrea et al., 2006), was interpreted as an evidence of the alteration of the stable endemy prevalent in this region, and an indication of the risk of outbreak of babesiosis by *B. bigemina*. Although there is a strong correlation between high antibody prevalence and endemic stability and between low antibody prevalence and endemic instability, the serological methods do not consistently detect carrier animals. Considering that in present study the detection of *B. bigemina* was based on PCR methods, the prevalence obtained refers to the presence of current infection and not to a simply exposure to the protozoan, as is the case of serological methods (Wagner et al., 1992). Therefore, it was possible to verify that, in this region, animals remain persistently infected and constituting potential sources of infection for the tick vectors, even those ones of genetic constitution considered more resistant to *Babesia* infections, such as pure Nelore.

According to Mahoney and Ross (1972), in endemically stable situations the age incidence of *B. bigemina* parasitemia rises from zero at birth to a maximum reach in between 6 and 24 months of age and then falls back to zero in older animals. Data obtained in the present study reinforces these observations, since *B. bigemina* merozoites were detected exclusively in calves, aged from 8 to 12 months, and were absent in cows. Besides this, in calves, most of infections (51/60) were detected by PCR reaction, while in cows, the majority (51/60) were detected by nPCR. Although the methodology used for the detection of *B. bigemina* (PCR and nPCR) do not allow a quantitative analysis of the DNA availability, analytic sensibility of the nPCR reactions utilized in the present study was estimated as being 100 times greater

than that of the PCR (Oliveira-Sequeira et al., 2005). So, it is possible to consider the existence of a higher availability of *B. bigemina* DNA in blood of PCR-positive animals than in nPCR-positive ones.

In the present study, calves were responsible for the infection of 72% of the ticks, proportion which is very similar to that obtained in a previous study in which crossbred (5/8 *B. taurus* + 3/8 *B. indicus*) dairy calves were responsible for 74% of the infected ticks (Oliveira-Sequeira et al., 2005). In both investigations, number of ticks infected on calves was significantly higher than that of ticks infected on cows. This observation is an indirect evidence of the greater availability of *B. bigemina* in the blood of calves. Importance of density of parasites in blood of vertebrate hosts is widely admitted for establishment of *Babesia* infection in ticks, both relating to *B. bigemina* (Riek, 1964; Callow, 1968; Oliveira-Sequeira et al., 2005) and to the other species of *Babesia* (Riek, 1966; Yeruham et al., 2001).

It is important to note that the frequency of *B. bigemina* infection in the ticks fed on pure *B. indicus* cattle was similar to that fed on *B. taurus* × *B. indicus* cattle, revealing no difference among the genetic groups analyzed. On the other hand, these frequencies were significantly lower than those observed in ticks fed on cattle with predominant *B. taurus* infusion (Oliveira-Sequeira et al., 2005). Considering that reduced transmission capacity of *Babesia* spp. has been reported in ticks attached to resistant animals (Francis and Litte, 1964; Mahoney et al., 1981), we may suppose that the greater the contribution of European breeds to the genotype of an animal, the higher is its ability to transmit *B. bigemina* to ticks.

Occurrence of endemic stability is claimed to be the main factor in limiting losses due to tick-born diseases, and disruption of this complex relationship might result in an increase in clinical disease incidence (Coleman et al., 2001). In the case of bovine babesiosis, rates of babesial infection in cattle and ticks are modulated by climate, soil, and cattle biotypes (Guglielmone, 1995). Considering the data presented here, we may suppose that under suitable abiotic conditions to tick development, the state of endemic stability could not be disrupted by resistant cattle biotypes, confirming previous report, that in Brazil (Lima et al., 2000), the maintenance of endemic stability of *B. bigemina* requires a minimum of tick challenge.

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